DNA FRAGMENT CARRYING TOLUENE MONOOXYGENASE GENE, RECOMBINANT PLASMID, TRANSFORMED MICROORGANISM, METHOD FOR DEGRADING CHLORINATED ALIPHATIC HYDROCARBON COMPOUNDS AND AROMATIC COMPOUNDS, AND METHOD FOR ENVIRONMENTAL REMEDIATION

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a novel DNA fragment carrying a toluene monooxygenase gene, a novel recombinant DNA containing the DNA fragment, a transformant containing the recombinant DNA, and a method for degrading chlorinated aliphatic hydrocarbon compounds such as trichloroethylene (TCE) and dichloroethylene (DCE) and aromatic compounds such as toluene, benzene, phenol, and cresol. The present invention also relates to a method for environmental remediation useful for cleaning of aqueous media such as wastewater and effluent containing at least either a chlorinated aliphatic hydrocarbon compound or an aromatic compound and air (gas phase) and soil polluted with chlorinated aliphatic hydrocarbon compounds.

Related Background Art

Recently, it has become a serious problem the environmental pollution with volatile organic chlorinated compounds which are harmful to the organisms and hardly degradable. Especially, the soil

in the industrial areas in Japan as well as abroad is considered to be contaminated with chlorinated aliphatic hydrocarbon compounds such as tetrachloroethylene (PCE), trichloroethylene (TCE), and dichloroethylene (DCE) and aromatic compounds such as toluene, benzene, phenol, and cresol. There have been a number of reports on actual detection of such pollutants through environmental surveys. It is supposed that these compounds remaining in soil dissolve in ground water via rainwater, and thereby spread over the surrounding areas. There is a strong suspicion that these compounds are carcinogens, and further, these are quite stable in the environment; therefore contamination of groundwater, which is used as a source of drinking water, has become a serious social problem. Therefore, cleaning of aqueous media such as contaminated groundwater and soil through removal and degradation of these compounds and accompanying cleaning of the surrounding gas phase are quite important in view of the environment protection. Technologies required for cleaning (for example, adsorption treatment using activated carbon, degradation treatment using light and heat) have been developed. Technologies presently available, however, are not always practical in terms of cost and operability. Recently, microbial degradation of chlorinated aliphatic hydrocarbon compounds such as TCE

that is stable in environment has been reported. The microbial degradation method have advantages such as: (1) degradation of chlorinated aliphatic hydrocarbon compounds into harmless substances by using appropriately selected microorganism; (2) no requirement for any special chemicals in principle; and (3) reduction of the labor and costs of maintenance.

The examples of microorganisms capable of degrading TCE are as follows:
Welchia alkenophila sero 5 (U.S. Patent No. 4877736, ATCC 53570, Welchia alkenophila sero 33 (U.S. Patent No. 4877736, ATCC 53571), Methylocystis sp. Strain M (Agric. Biol. Chem., 53, 2903 (1989), Biosci. Biotech. Bichem., 56, 486 (1992), ibid. 56, 736 (1992)), Methylosinus trichosporium OB3b (Am. Chem. Soc. Natl. meet. Div. Environ. Microbiol., 29, 365 (1989), Appl. Environ. Microbiol., 55, 3155 (1989), Appl. Biochem. Biotechnol. 28, 877 (1991), Japanese Patent Application Laid-Open No. 2-92274 specification, Japanese Patent Laid-Open Application No. 3-292970), Methylomonas sp. MM2 (Appl. Environ. Microbiol., 57, 236 (1991), Alcaligenes denitrificans ssp. Xylosoxidans JE75 (Arch. Microbiol., 154, 410 (1990), Alcaligenes eutrophus JMP134 (Appl. Environ. Microbiol., 56, 1179 (1990), Alcaligenes eutrophus FERM-13761 (Japanese Patent Laid-Open Application No. 7-123976), Pseudomonas

aeruginosa J1104 (Japanese Patent Application Laid-Open No. 7-236895), Mycobacterium vaccae JOB5 (J. Gen. Microbiol., 82, 163 (1974), Appl. Environ. Microbiol., 55, 2960 (1989), ATCC 29678), Pseudomonas putida BH (Gesuidou Kyoukai-shi (Japan Sewage Works Association Journal), 24, 27 (1987)), Pseudomonas sp. strain G4 (Appl. Environ. Microbiol., 52, 383 (1968), ibid. 53, 949 (1987), ibid. 54, 951 (1988), ibid. 56, 279 (1990), ibid. 57, 193 (1991), U.S. Patent No. 4925802, ATCC 53617, this strain was first classified as Pseudomonas cepacia and then changed to Pseudomonas sp.), Pseudomonas mendocia KR-1 (Bio/Technol., 7, 282 (1989)), Pseudomonas putida F1 (Appl. Environ Microbiol., 54, 1703 (1988), ibid. 54, 2578 (1988)), Pseudomonas fluorescens PFL12 (Appl. Environ. Microbiol., 54, 2578 (1988)), Pseudomonas putida KWI-9 (Japanese Patent Application Laid-Open No. 6-70753), Pseudomonas cepacia KK01 (Japanese Patent Application Laid-Open No. 6-22769), Nitrosomonas europaea (Appl. Environ. Microbio., 56, 1169 (1990), Lactobacillus vaginalis sp. nov (Int. J. Syst. Bacteriol., 39, 368 (1989), ATCC 49540), Nocardia corallina B-276 (Japanese Patent Application Laid-Open No. 8-70881, FERM BP-5124, ATCC 31338), and so on.

The problem in actually using these degrading microorganisms in environmental remediation treatment, however, resides in optimizing and maintaining

expression of their degradation activity for chlorinated aliphatic hydrocarbon compounds such as TCE. In an environmental remediation treatment which utilizes phenol, toluene, methane, or the like as an inducer, continuous supply of the inducer is indispensable, since depletion of such inducers directly results in stoppage of degradation of chlorinated aliphatic hydrocarbon compounds. Presence of such inducers, on the other hand, may inhibit the efficient degradation of the target substance such as TCE, since the affinity of the chlorinated aliphatic hydrocarbon compounds such as TCE as a substrate is considerably low in comparison with these inducers. In addition, precise control of the inducer concentration on the treatment spot is difficult.

Thus, use of an inducer is a large problem in practical application of environmental remediation treatment utilizing microorganisms.

In order to solve the problem, Nelson et al. developed a method using tryptophan as an inducer for degradation of volatile organic chlorinated compounds (Japanese Patent Application Laid-Open No. 4-502277). Tryptophan, however, is a very expensive substance, and although tryptophane has no toxicity or risk as a substance, it is not preferable to introduce excessive carbon and nitrogen sources into environment since it may induce eutrophication. In addition, the problem

that tryptophan serves as a competitive inhibitor in degradation of TCE still remains.

Shields et al. obtained a mutant strain of Pseudomonas cepacia G4 (changed to Pseudomonas sp. upon deposition to ATCC) by the transposon technique, which mutant strain does not require an inducer (in this case, phenol or toluene) and can degrade TCE (Appl. Environ. Microbiol., 58, 3977 (1992), International Publication No. WO/19738). Also, a mutant not requiring methane as the inducer has been isolated from Methylosinus trichosporium OB3b, a methanotroph capable of degrading TCE (U.S. Patent No. 5316940).

Japanese Patent Application Laid-Open No. 8-294387 also discloses strain JM1 (FERM BP-5352) capable of degrading volatile organic chlorinated compounds and aromatic compounds without requiring an inducer, isolated by nitrosoguanidine mutagenization of strain J1 (FERM BP-5102). While, it has been studied to introduce resting cells expressing TCE-degrading activity into the remediation site after the preculture of the cells in the presence of an inducer (Environ. Sci. Technol., 30, 1982 (1996)).

It has been reported that remediation treatment not requiring the inducer actually makes the remediation treatment easy and efficient compared to the conventional treatment using inducers.

However, the growth control of the degrading microorganisms is very important for both the expression of the degradation activity on demand and the continuation of degradation. When resting cells are used, it is a problem to be solved that TCE cannot be degraded beyond the amount and period of degradation capacity of the introduced resting cells. In addition, in a large scale treatment, there are further problems that degradation activity will decrease since it takes a long time to prepare resting cells; the treating apparatus must be large in scale; treatment process is complicated; and the cost may be unfavorably high. Accordingly, it has been attempted to introduce a plasmid carrying a DNA fragment containing a gene region encoding oxygenase or hydroxylase into a host microorganism to make the host express the TCE degradation activity constitutively or inducibly using a harmless inducer. For example, there are Pseudomonas mendocina KR-1 (Japanese Patent Application Laid-Open No. 2-503866, Pseudomonas putida KWI-9 (Japanese Patent Application Laid-Open No. 6-105691), Pseudomonas putida BH (Summary of 3rd Conference on Pollution of Ground Water/Soil and Its Protective Countermeasure, p.213 (1994)), and a transformant carrying both a toluene degradation enzyme gene derived from Pseudomonas putida F1 and a biphenyl degradation enzyme gene derived from Pseudomonas pseudoalkaligenes

(Japanese Patent Application Laid-Open No. 7-143882). However, the reported TCE degradation activity of the transformants are low, and the advantages of the transformants has not been fully utilized for efficient degradation of TCE, such as the ease of degradation control, freedom in designing recombinant, and no requirements for inducers.

SUMMARY OF THE INVENTION

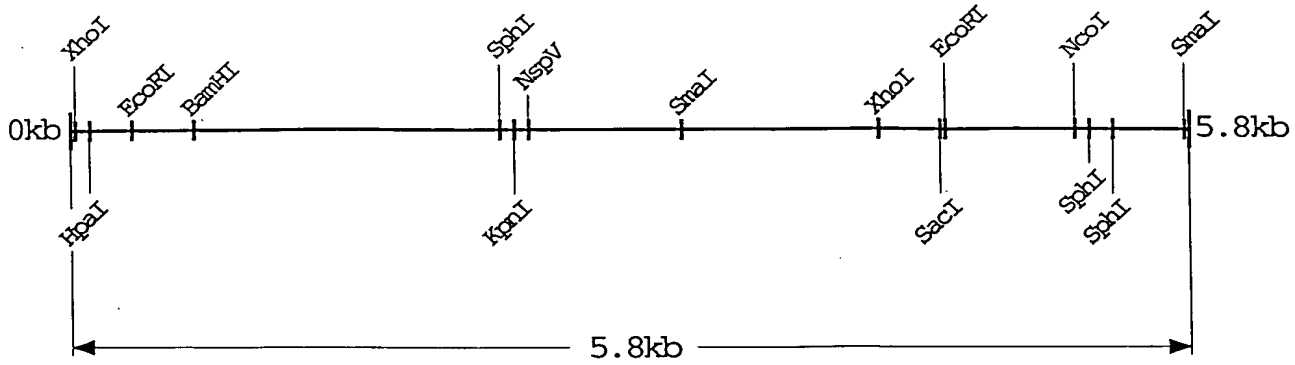
It is an object of the present invention to provide a novel DNA fragment encoding a toluene monooxygenase of a high efficiency in degrading aromatic compounds and/or organic chorine compounds, a novel recombinant DNA containing the DNA fragment, and a transformant containing the recombinant DNA. It is another object of the present invention to provide an efficient biodegradation method for volatile organic chlorinated compounds such as trichloroethylene (TCE) and dichloroethylene (DCE) and aromatic compounds such as toluene, benzene, phenol, and cresol using the transformant, specifically an efficient environmental remediation method useful for purifying aqueous media such as wastewater and effluent containing chlorinated aliphatic hydrocarbon compounds or aromatic compounds, remedying soil polluted with chlorinated aliphatic hydrocarbon compounds or aromatic compounds, and purifying air (gas phase) polluted with chlorinated

aliphatic hydrocarbon compounds.

To achieve the above objects, the inventors of the present invention strained to isolate the gene encoding toluene monooxygenase from Burkholderia cepacia KK01 (previously Pseudomonas cepacia, deposited in the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology in accordance with the requirements of the Budapest Treaty, Deposit Date: March 11, 1992, Accession No. FERM BP-4235) having a toluene monooxygenase that oxidizes toluene to ortho-cresol and 3-methylcatechol. Successful isolation and characterization of the gene completed the present invention.

According to one aspect of the present invention, there is provided a DNA fragment of about 5.8 Kb containing a toluene monooxygenase gene having a following restriction map, where 1 BamHI restriction site, 2 EcoRI restriction sites, 1 HpaI restriction site, 1 KpnI restriction site, 1 NcoI restriction site, 1 NspV restriction site, 1 SacI restriction site, 2 SmaI restriction sites, 3 SphI restriction sites, 2 XhoI restriction sites, no ClaI restriction site, no DraI restriction site, no EcoRV restriction site, no HindIII restriction site, no NdeI restriction site, no NheI restriction site, no PvuII restriction site, no ScaI restriction site, no Sse8387I restriction site, no StuI restriction site, and no XbaI restriction site are

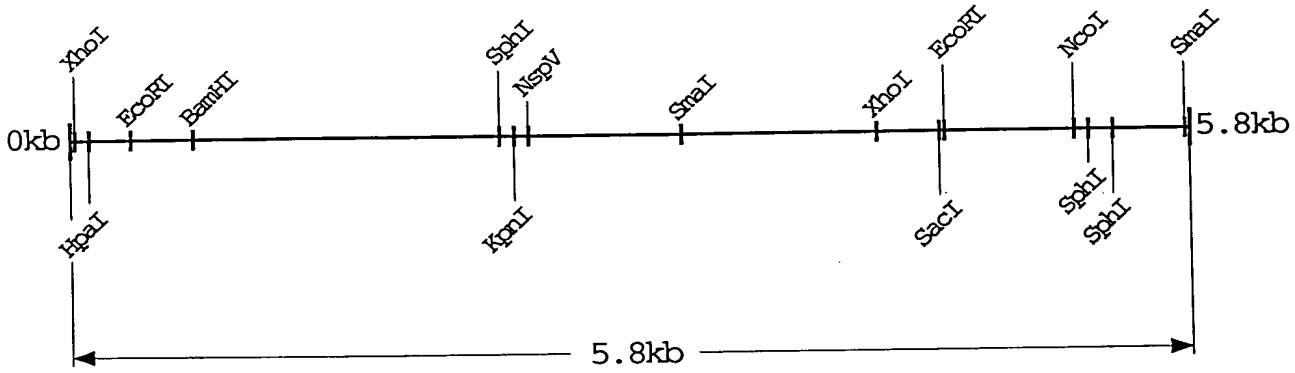
present.



According to another embodiment of the present invention, there is provided a DNA fragment having the nucleotide sequence of SEQ ID NO: 1 with deletion, substitution and/or addition of one or more nucleotides, still encoding an active toluene monooxygenase.

Further, according to one aspect of the present invention, there is provided a recombinant DNA comprising a vector enabling maintenance or replication in a host and a DNA fragment of about 5.8 Kb containing a toluene monooxygenase gene having a following restriction map, where 1 BamHI restriction site, 2 EcoRI restriction sites, 1 HpaI restriction site, 1 KpnI restriction site, 1 NcoI restriction site, 1 NspV restriction site, 1 SacI restriction site, 2 SmaI restriction sites, 3 SphI restriction sites, 2 XhoI restriction sites, no ClaI restriction site, no DraI

restriction site, no EcoRV restriction site, no HindIII restriction site, no NdeI restriction site, no NheI restriction site, no PvuII restriction site, no ScaI restriction site, no Sse8387I restriction site, no StuI restriction site, and no XbaI restriction site are present.



Further, according to another embodiment of the present invention, there is provided another recombinant DNA comprising a vector enabling maintenance or replication in a host, and a DNA fragment ligated thereto having the nucleotide sequence of SEQ ID NO: 1 with deletion, substitution and/or addition of one or more bases, still encoding an active toluene monooxygenase.

According to still another aspect of the present invention, there is provided another recombinant DNA comprising a vector enabling maintenance or replication in a host, and a DNA fragment containing a region encoding a toluene monooxygenase, where the region

comprises a first sequence encoding a polypeptide TomL having an amino acid sequence of SEQ ID NO: 3, a second sequence encoding a polypeptide TomM having an amino acid sequence of SEQ ID NO: 4, a third sequence encoding a polypeptide TomN having an amino acid sequence of SEQ ID NO: 5, a fourth sequence encoding a polypeptide TomO having an amino acid sequence of SEQ ID NO: 6, and a fifth sequence encoding a polypeptide TomP having an amino acid sequence of SEQ ID NO: 7, and the first to fifth sequences are aligned so that expressed TomL - TomP can form an active monooxygenase protein.

According to still another aspect of the present invention, there is provided another recombinant DNA comprising a vector enabling maintenance or replication in a host, and a DNA fragment containing a region encoding a toluene monooxygenase, where the region comprises a first sequence encoding a polypeptide TomL having an amino acid sequence of SEQ ID NO: 3, a second sequence encoding a polypeptide TomM having an amino acid sequence of SEQ ID NO: 4, a third sequence encoding a polypeptide TomN having an amino acid sequence of SEQ ID NO: 5, a fourth sequence encoding a polypeptide TomO having an amino acid sequence of SEQ ID NO: 6, and a fifth sequence encoding a polypeptide TomP having an amino acid sequence of SEQ ID NO: 7, and the first to fifth sequences are aligned so that

expressed TomL - TomP can form an active monooxygenase protein, wherein one or more nucleotides have been deleted, substituted, or added in at least one of the sequences with the proviso that the activity of toluene monooxygenase is not impaired.

According to still another aspect of the present invention, there is provided a DNA fragment containing a region encoding a polypeptide TomK having an amino acid sequence of SEQ ID NO: 2 wherein the function of TomK is to enhance a toluene monooxygenase activity of a protein consisting at least of TomL to TomP, or encoding a variant TomK having an amino acid sequence varied from SEQ ID NO: 2 with the proviso that the function of TomK is not impaired.

According to still another aspect of the present invention, there is provided a recombinant DNA comprising a vector; a promoter; and a DNA fragment containing a region encoding a toluene monooxygenase, where the region comprises a first sequence encoding a polypeptide TomL having an amino acid sequence of SEQ ID NO: 3, a second sequence encoding a polypeptide TomM having an amino acid sequence of SEQ ID NO: 4, a third sequence encoding a polypeptide TomN having an amino acid sequence of SEQ ID NO: 5, a fourth sequence encoding a polypeptide TomO having an amino acid sequence of SEQ ID NO: 6, and a fifth sequence encoding a polypeptide TomP having an amino acid sequence of SEQ

ID NO: 7, and the first to fifth sequences are aligned so that expressed TomL - TomP can form an active monooxygenase protein;

wherein the promoter is linked to the DNA fragment in a manner allowing expression of the toluene monooxygenase protein encoded by the DNA fragment.

According to still another aspect of the present invention, there is provided a recombinant DNA comprising a vector; a promoter; and a DNA fragment containing a region encoding a toluene monooxygenase, where the region comprises a first sequence encoding a polypeptide TomL having an amino acid sequence of SEQ ID NO: 3, a second sequence encoding a polypeptide TomM having an amino acid sequence of SEQ ID NO: 4, a third sequence encoding a polypeptide TomN having an amino acid sequence of SEQ ID NO: 5, a fourth sequence encoding a polypeptide TomO having an amino acid sequence of SEQ ID NO: 6, and a fifth sequence encoding a polypeptide TomP having an amino acid sequence of SEQ ID NO: 7, and the first to fifth sequences are aligned so that expressed TomL - TomP can form an active monooxygenase protein,

wherein one or more nucleotides have been deleted from, substituted in, and/or added to at least one of the sequences of the DNA fragment with the proviso that the protein does not loose toluene monooxygenase activity,

wherein the promoter and the DNA fragment are functionally linked in a manner enabling expression of the toluene monooxygenase protein encoded by the DNA fragment.

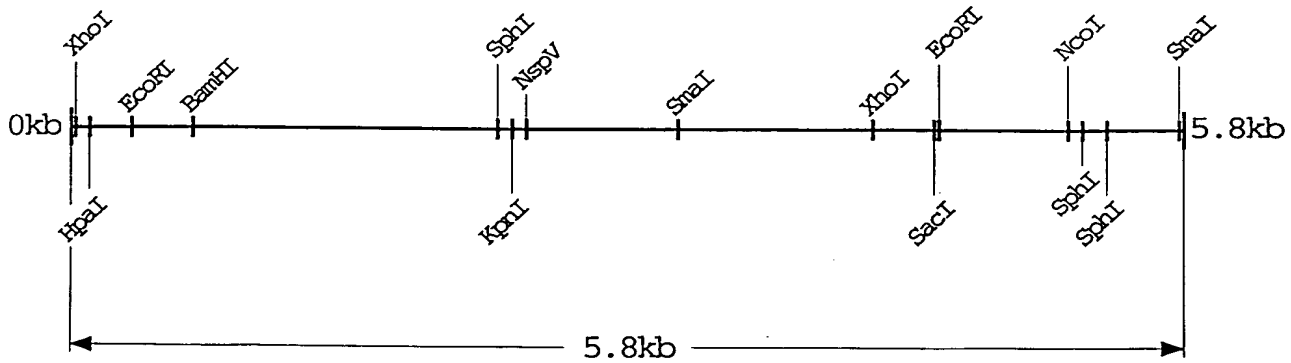
According to still another aspect of the present invention, there is provided a recombinant DNA comprising a vector; a first promoter and a first DNA fragment functionally linked thereto; and a second promoter and a second DNA fragment functionally linked thereto; wherein the first DNA fragment contains a region encoding a polypeptide TomK having an amino acid sequence of SEQ ID NO: 2 wherein the function of TomK is to enhance a toluene monooxygenase activity of a protein consisting at least of TomL to TomP, or encoding a variant TomK having an amino acid sequence varied from SEQ ID NO: 2 with the proviso that the function of TomK is not impaired; the second DNA fragment contains a region encoding a toluene monooxygenase, where the region comprises a first sequence encoding a polypeptide TomL having an amino acid sequence of SEQ ID NO: 3, a second sequence encoding a polypeptide TomM having an amino acid sequence of SEQ ID NO: 4, a third sequence encoding a polypeptide TomN having an amino acid sequence of SEQ ID NO: 5, a fourth sequence encoding a polypeptide TomO having an amino acid sequence of SEQ ID NO: 6, and a fifth sequence encoding a polypeptide TomP having an

amino acid sequence of SEQ ID NO: 7, and the first to fifth sequences are aligned so that expressed TomL - TomP can form an active monooxygenase protein, wherein one or more nucleotides have been deleted from, substituted in, and/or added to at least one of the sequences of the second DNA fragment with the proviso that the protein does not loose toluene monooxygenase activity,

wherein the vector is linked to the DNA fragment in a manner enabling expression of the toluene monooxygenase protein encoded by the DNA fragment.

Further, according to still another aspect of the present invention, there is provided a transformant obtainable by introducing a recombinant DNA comprising a vector enabling maintenance or replication in a host and a DNA fragment of about 5.8 Kb containing a toluene monooxygenase gene having a following restriction map, where 1 BamHI restriction site, 2 EcoRI restriction sites, 1 HpaI restriction site, 1 KpnI restriction site, 1 NcoI restriction site, 1 NspV restriction site, 1 SacI restriction site, 2 SmaI restriction sites, 3 SphI restriction sites, 2 XhoI restriction sites, no ClaI restriction site, no DraI restriction site, no EcoRV restriction site, no HindIII restriction site, no NdeI restriction site, no NheI restriction site, no PvuII restriction site, no ScaI restriction site, no Sse8387I restriction site, no StuI restriction site,

and no XbaI restriction site are present.



Further, according to still another aspect of the present invention there is provided a transformant obtainable by introducing a recombinant DNA into a host microorganism, where the recombinant DNA comprises a vector enabling maintenance or replication in a host, and a DNA fragment ligated thereto having the nucleotide sequence of SEQ ID NO: 1 with deletion, substitution and/or addition of one or more bases, still encoding an active toluene monooxygenase.

Further, according to still another aspect of the present invention, there is provided a transformant obtainable by introducing a recombinant DNA comprising a vector, a promoter and a DNA fragment into a host microorganism where the DNA fragment contains a region encoding a toluene monooxygenase, where the region comprises a first sequence encoding a polypeptide TomL having an amino acid sequence of SEQ ID NO: 3, a second sequence encoding a polypeptide TomM having an amino

acid sequence of SEQ ID NO: 4, a third sequence encoding a polypeptide TomN having an amino acid sequence of SEQ ID NO: 5, a fourth sequence encoding a polypeptide TomO having an amino acid sequence of SEQ ID NO: 6, and a fifth sequence encoding a polypeptide TomP having an amino acid sequence of SEQ ID NO: 7, and the first to fifth sequences are aligned so that expressed Tom L - TomP can form an active monooxygenase protein;

wherein the promoter and the DNA fragment are functionally linked in a manner enabling expression of the toluene monooxygenase protein encoded by the DNA fragment.

According to still another aspect of the present invention, there is provided a method for producing a toluene monooxygenase, which comprises a step of making the transformant according to any one of the embodiment of the present invention mentioned above to produce the toluene monooxygenase being a gene product of the recombinant DNA introduced in the transformant.

According to still another aspect of the present invention, there is provided a method for degrading at least either of a chlorinated aliphatic hydrocarbon compound or an aromatic compound, which comprises a step of degrading at least either of the chlorinated aliphatic hydrocarbon compound or aromatic compound using the transformant according to any one of the

aspects of the present invention mentioned above.

According to still another aspect of the present invention, there is provided a method for cleaning a medium contaminated with at least either of a chlorinated aliphatic hydrocarbon compound or an aromatic compound, which comprises a step of degrading at least either of a chlorinated aliphatic hydrocarbon compound or an aromatic compound using the transformants according to any one of the aspects of the present invention mentioned above.

According to still another aspect of the present invention, there is provided a method of remedying an environment polluted with at least either of a chlorinated aliphatic hydrocarbon compound or an aromatic compound as a pollutant, comprising a step of degrading the pollutants using the transformant according to any one of the aspects of the present invention mentioned above.

According to still another aspect of the present invention, there is provided a component polypeptide having any one of amino acid sequences of SEQ ID Nos: 2-8, which can constitute a toluene monooxygenase.

According to still another aspect of the present invention, there is provided a toluene monooxygenase comprising at least component polypeptides TomL-TomP of amino acid sequences of SEQ ID NOs: 3-7.

According to still another aspect of the present

invention, there is provided a variant toluene monooxygenase comprising at least component polypeptides TomL-TomP of amino acid sequences of SEQ ID Nos.: 3-7 wherein one or more amino acids have been deleted from, substituted to, and/or added to the polypeptides with the proviso that the toluene monooxygenase does not loose its activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a restriction map of a DNA fragment of about 5.8 Kb carrying a toluene monooxygenase gene;

Fig. 2 is comprised of Figs. 2A, 2B, 2C, 2D, 2E, 2F, 2G, 2H, 2I, 2J, 2K, 2L, 2M, 2N, 2O, 2P, 2Q and 2R showing a nucleotide sequence of a toluene monooxygenase gene of FERM BP-4235;

Fig. 3 is an amino acid sequence (TomK) encoded by a region tomK in the nucleotide sequence of Fig. 2;

Fig. 4 is comprised of Figs. 4A, 4B and 4C showing an amino acid sequence (TomL) coded by a region tomL in the nucleotide sequence of Fig. 2;

Fig. 5 is an amino acid sequence (TomM) coded by a region tomM in the nucleotide sequence of Fig. 2;

Fig. 6 is comprised of Figs. 6A, 6B, 6C and 6D showing an amino acid sequence (TomN) coded by a region tomN in the nucleotide sequence of Fig. 2;

Fig. 7 is an amino acid sequence (TomO) coded by a region tomO in the nucleotide sequence of Fig. 2;

Fig. 8 is comprised of Figs. 8A, 8B and 8C showing an amino acid sequence (TomP) coded by a region tomP in the nucleotide sequence of Fig. 2;

Fig. 9 is an amino acid sequence (TomQ) coded by a region tomQ in the nucleotide sequence of Fig. 2;

Fig. 10 is a nucleotide sequence of a first primer employed in Example 6;

Fig. 11 is a nucleotide sequence of a second primer employed in Example 6;

Fig. 12 is a nucleotide sequence of a third primer employed in Example 6;

Fig. 13 is a nucleotide sequence of a fourth primer employed in Example 6;

Fig. 14 is a nucleotide sequence of a fifth primer employed in Example 6; and

Fig. 15 shows time-course changes in TCE in the gas phase in Example 3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The DNA fragment containing a toluene monooxygenase gene according to the present invention is isolated from Burkholderia cepacia strain KK01 (FERM BP-4235, hereinafter referred to as Strain KK01). The microbiological characteristics and culture conditions of Strain KK01 are as follows (see Japanese Patent Application Laid-Open No. 6-22769).

Strain KK01

· Morphological characteristics

(1) Gram staining: Negative

(2) Size and shape: Rod of 1.0-2.0 μm in length and 0.5 μm in width

(3) Motility: Motile

B. Growth on various culture media

Medium	Growth temperature (°C)	Growth
Blood agar medium	37	+
Lactose agar medium	37	+
Chocolate agar medium	37	++
GMA	37	-
Scylo	37	-
Standard agar medium	4	-
Standard agar medium	25	±
Standard agar medium	37	+
Standard agar medium	41	±

C. Physiological characteristics

(1) Aerobic or anaerobic: Obligate aerobic

(2) Sugar degradation mode: Oxidation

(3) Oxidase production: +

(4) Silver nitrate reduction: +

(5) Hydrogen sulfide production: -

(6) Indole production: -

(7) Urease production: -

(8) Gelatin liquefaction: -

(9) Arginine hydrolysis: -

(10) Lysine decarboxylation: +

(11) Ornithine decarboxylation: -

(12) Utilization of citric acid: +

(13) Methyl carbinol acetyl reaction (VP reaction): -

(14) Detection of tryptophan deaminase: -

(15) ONPG:

(16) Assimilation of carbohydrates

Glucose: +

Fructose: +

Maltose: +

Galactose: +

Xylose: +

Mannitol: ±

Sucrose: -

Lactose: +

Esculin: -

Inositol: -

Sorbitol: -

Rhamnose: -

Melibiose: -

Amygdalin: -

L-(+)-arabinose: +

Isolation of the DNA fragment according to the present invention is achieved by partial digestion of the total DNA of strain KK01 with a restriction enzyme Sau3AI. Specifically, total DNA can be prepared by the standard method, in which the above microorganism is grown in a suitable medium, for example, LB medium

(containing 10 g of trypton, 5 g of yeast extract, and 5 g of sodium chloride in 1 litter) and then cells are disrupted, for example, in the presence of sodium dodecyl sulfate (SDS) at 70°C. The total DNA is then partially digested by Sau3AI to obtain a DNA fragment of about 5.8 Kb carrying a toluene monooxygenase gene. The DNA fragment thus obtained is ligated to a plasmid vector completely digested by BamHI, for example, pUC18, and the recombinant vector is introduced into competent cells of, for example, E. coli JM109, prepared by the Hanahan method to obtain transformants. Then, transformants can be selected by a suitable method, for example, by culturing cells on an LB medium plate containing ampicillin.

In order to select a transformant containing a recombinant vector carrying a toluene monooxygenase gene from the above transformants, it is preferable to add cresol, phenol, or the like to LB medium for transformant selection in advance. The transformant carrying a toluene monooxygenase gene can be selected as brown colonies, since these substrates are monooxygenated by toluene monooxygenase to produce methylcatechol or catechol which is then autooxidized to develop color. Alternatively, after culturing cells on an ordinary LB medium plate, various substrates may be sprayed onto the plate to select brown colonies in a similar manner.

The isolated DNA fragment of about 5.8 Kb has the following restriction sites:

Restriction enzyme	Number of restriction sites
BamHI	1
EcoRI	2
HpaI	1
KpnI	1
NcoI	1
NspV	1
SacI	1
SmaI	2
SphI	3
XhoI	2

The DNA fragment has no ClaI, DraI, EcoRV, HindIII, NdeI, NheI, PvuII, ScaI, Sse8387I, StuI, or XbaI restriction site.

The restriction map of the DNA fragment of the present invention is as shown above. Toluene monooxygenase genes derived from Burkholderia cepacia G4 5223 PR1 (U.S. Patent No. 5543317), derived from Burkholderia sp. JS150 (Appl. Environ. Microbiol., 61,

3336 (1995), derived from Pseudomonas pickettii PK01 (J. Bacteriol., 176, 3749 (1994)), and derived from Pseudomonas mendocina KR1 (J. Bacteriol., 173, 3010 (1991)) were reported. Phenol hydroxylases reported to have a similar structure are derived from Acinetobacter calcoaceticus NCIIB8250 (Mol. Microbiol., 18, 13 (1995)), Pseudomonas sp. CF600 (J. Bacteriol., 172, 6826 (1990)), Pseudomonas spp. (J. Bacteriol., 177, 1485 (1995)), and Pseudomonas putida P35X (Gene, 151, 29 (1994)). The DNA fragment of the present invention has, however, a restriction map different from any of those. It is thus clear that the DNA fragment of the present invention contains a novel toluene monooxygenase gene.

Although the DNA fragment thus obtained can sufficiently enables the degradation of aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds even in pUC18, it can be integrated in an expression vector or a vector of a wide host range to improve the degradation ability or to be optimized for the treatment site.

The plasmid according to the present invention can be constructed from following elements:

1) Toluene monooxygenase gene;

2) Marker gene (drug-resistance, auxotrophic complement, or the like); and

3) Vector containing an autonomous replication

sequence (plasmid, or the like).

As the toluene monooxygenase gene, the DNA fragment of about 5.8 kb as shown above can be employed by itself, or a constitution containing elements necessary for a toluene monooxygenase activity can be also employed, for example, with or without spacer sequences. Further, each element can be varied with the proviso that its function is not impaired. These variations can be attained by changing DNA sequences encoding them.

As the drug-resistance genes, an ampicillin resistance gene, a kanamycin (G418, neomycin) resistance gene, a tetracycline resistance gene, a chloramphenicol resistance gene, a hygromycin resistance gene can be employed. For auxotrophic complement, a gene sequence to supply the nutrient required by the host organism is used. Typically, a gene enabling the synthesis of the required amino acid is utilized.

As the autonomous replication sequences, a sequence derived from plasmid RSF1010, which can function as a wide host range replication region in most of the gram-negative bacteria, can be employed. It can be also employed vector pBBR122 (Mo Bi Tec) containing a wide host range replication region which does not belong to any incompatible groups, IncP, IncQ, or IncW or the like.

For the recombinant plasmid according to the present invention, various promoters and terminators can be employed and various factors can be further introduced to improve and control the ability of degrading aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds. Specifically, promoters such as lac, trc, tac, T3, and T7 can be employed. As a terminator, a rrnB operon terminator or the like can be employed. Also, introduction of a repressor gene such as lacIq and a lac operator enables expression control with an inducer such as isopropyl thiogalactoside (IPTG). Alternatively, the absence of these suppressor and operator as elements, enables constitutive expression of degradation activity. In addition, a temperature-sensitive control system or the like can be employed.

For recombination of a DNA fragment containing the toluene monooxygenase gene into an expression vector containing these regulating elements, natural restriction sites can be utilized as it is, or restriction sites may be newly created by site-directed mutagenesis or a polymerase chain reaction using a primer involving base substitution. In general, recombination into an expression vector often utilizes NcoI restriction sites. It is convenient to design so as to create an NcoI restriction site in the initiation codon ATG or GTG region by site-directed mutagenesis or

primer design. Known methods using an adaptor can be employed. For optimization of expression, the DNA fragment may be properly deleted using exonuclease III or Bal31 nuclease. As described above, molecular biological techniques suitable for the purpose can be employed for recombination into an expression vector.

As a method for introducing the recombinant plasmid carrying a desired gene into a host organism, any methods that can introduce a foreign gene into a host can be employed, and known methods, for example, the calcium chloride method, the electroporation method, and the conjugation transfer method can be employed.

In the present invention, any microorganisms can be used as a host organism so long as it can express the aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds-degrading activity after the introduction of the recombinant plasmid, including the genera Escherichia, Pseudomonas, Burkholderia, Acinetobacter, Moraxella, Alcaligenes, Vibrio, Nocardia, Bacillus, Lactobacillus, Achromobacter, Arthrobacter, Micrococcus, Mycobacterium, Methylosinus, Methylomonas, Welchia, Methylocystis, Nitrosomonas, Saccharomyces, Candida, Torulopsis, and Ralstonia.

In addition, the aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds-degrading microorganisms such as strain J1, strain JM1,

Pseudomonas sp. strain TL1, strain KK01, Pseudomonas alcaligenes strain KB2, Alcaligenes sp. strain TL2, and Vibrio sp. strain KB1 can be employed as a host. These strains have been deposited in the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology of Japan. The date of deposit, Accession No., and microbiological characteristics of these strains other than the strain KK01 already described are shown below.

Strain J1 (Deposit date: May 25, 1994, Accession No. FERM BP-5102)

A. Morphological characteristics

Gram staining: Positive

Size and shape of cells: Polymorphous rod of 1-6 μm in length and about 0.5-2 μm in width

Mobility: Negative

Colony: Cream to light pink, sticky

B. Growth on various media

BHIA: Good growth

MacConkey: No growth

C. Optimal temperature for growth: 25°C > 30°C > 35°C

D. Physiological characteristics

Aerobic or anaerobic: aerobic

TSI (slant/butt): Alkaline/alkaline, H_2S (-)

Oxidase: Negative

Catalase: Positive

Sugar fermentation

Glucose: Negative

Sucrose: Negative

Raffinose: Negative

Galactose: Negative

Maltose: Negative

Urease: Positive

Esculin: Positive

Nitric acid: Negative

Strain JM1 (Deposit date: January 10, 1995, Accession No. FERM BP-5352)

Gram staining and morphology: Gram-negative rod

Growth on various media

BHIA: Good growth

MacConkey: Possible to grow

Colony color: Cream

Optimal temperature for growth: 25°C > 30°C > 35°C

Mobility: Negative (semi-fluid medium)

TSI (slant/butt): Alkaline/alkaline, H_2S (-)

Oxidase: Positive (weak)

Catalase: Positive

Sugar fermentation

Glucose: Negative

Sucrose: Negative

Raffinose: Negative

Galactose: Negative

Maltose: Negative

Urease: Positive

Esculin hydrolysis (β-glucosidase): Positive

Nitrate reduction: Negative

Indole production: Negative

Glucose acidification: Negative

Arginine dehydrase: Negative

Gelatin hydrolysis (protease): Negative

β-Galactosidase: Negative

Assimilation of compounds

Glucose: Negative

L-Arabinose: Negative

D-Mannose: Negative

D-Mannitol: Negative

N-Acetyl-D-glucosamine: Negative

Maltose: Negative

Potassium gluconate: Negative

n-Capric acid: Positive

Adipic acid: Negative

dl-Malic acid: Positive

Sodium citrate: Positive

Phenyl acetate: Negative

Strain J1 is an aromatic compound-assimilating

bacterium which degrades organic chlorinated compounds with the participation of oxygenase. In spite of its excellent ability of degrading organic chlorinated compounds that it can almost completely degrade about 20 ppm of TCE at a low temperature of 15°C close to natural environment such as soil, it requires aromatic compounds such as phenol, toluene, and cresol as a degradation inducer. Strain JM1 has the same microbiological characteristics as the parental strain J1 except that it can degrade organic chlorinated compounds in the absence of aromatic compounds such as phenol, toluene, and cresol as a degradation inducer.

Strain TL1 (Deposit date: January 10, 1995, Deposit No. FERM P-14726/FERM BP-6923.

A. Gram staining and morphology: Gram-negative rod

B. Growth on various media

Standard agar: Good growth

MacConkey agar: Poor growth

C. Optimal temperature for growth: 25°C > 35°C

D. Physiological characteristics

Aerobic/anaerobic: Aerobic

TSI (slant/butt): Alkaline/alkaline, H_2S (-)

Oxidase: Positive

Catalase: Positive

Oxidation/fermentation test: -/-

Potassium nitrate reduction: Negative

Indole production from L-tryptophan: Negative

Glucose acidification: Negative

Arginine dehydrase: Negative

Urease: Negative

Esculin hydrolysis (β-glucosidase): Negative

Gelatin hydrolysis (protease): Negative

β-Galactosidase: Negative

Cytochrome oxidase: Positive

E. Assimilation of sugars, organic acids, etc.

Glucose: Positive

L-Arabinose: Positive

D-Mannose: Negative

D-Mannitol: Positive

N-Acetyl-D-glucosamine: Negative

Maltose: Negative

Potassium gluconate: Positive

n-Capric acid: Negative

Adipic acid: Positive

dl-Malic acid: Negative

Sodium citrate: Negative

Phenyl acetate: Negative

Strain TL2 (Deposit date on November 15, 1994, Deposit No. FERM P-14642/FERM BP-6913.

A. Gram staining and morphology: Gram-negative rod

B. Growth on various media

Standard agar: Good growth

MacConkey agar: Poor growth

C. Optimal temperature for growth: 25°C >35°C

D. Physiological characteristics

Aerobic/anaerobic: Aerobic

TSI (slant/butt): Alkaline/alkaline, H_2S (-)

Oxidase: Positive

Catalase: Positive

Oxidation/fermentation test: -/-

Potassium nitrate reduction: Positive

Indole production from L-tryptophan: Negative

Glucose acidification: Negative

Arginine dehydrase: Negative

Urease: Negative

Esculin hydrolysis (β-glucosidase): Negative

Gelatin hydrolysis (protease): Negative

β-Galactosidase: Negative

Cytochrome oxidase: Positive

E. Assimilation of sugars, organic acids, etc.

Glucose: Negative

L-Arabinose: Negative

D-Mannose: Negative

D-Mannitol: Negative

N-Acetyl-D-glucosamine: Negative

Maltose: Negative

Potassium gluconate: Positive

n-Capric acid: Positive

Adipic acid: Positive

dl-Malic acid: Positive

Sodium citrate: Positive

Phenyl acetate: Positive

Strain KB1 (Deposit date: November 15, 1994, Deposit No. FERM P-14643/FERM BP-6914.

A. Gram staining and morphology: Gram-negative bacillus

B. Growth conditions on various media

Standard agar: Good growth

MacConkey agar: Good growth

C. Optimal temperature for growth: 25°C > 35°C

D. Physiological characteristics

Aerobic/anaerobic: Aerobic

TSI (slant/butt): Alkaline/alkaline, H2 S(-)

Catalase: Positive

Oxidation/fermentation test: -/-

Potassium nitrate reduction: Positive

Indole productivity from L-tryptophan: Negative

Glucose acidification: Negative

Arginine dehydrase: Positive

Urease: Positive

Esculin hydrolysis (β-glucosidase): Negative

Gelatin hydrolysis (protease): Negative

β-Galactosidase: Negative

Cytochrome oxidase: Positive

E. Assimilation of sugars, organic acids, etc.

Glucose: Negative

L-Arabinose: Negative

D-Mannose: Negative

D-Mannitol: Negative

N-Acetyl-D-glucosamine: Positive

Maltose: Negative

Potassium gluconate: Positive

n-Capric acid: Positive

Adipic acid: Positive

dl-Malic acid: Positive

Sodium citrate: Negative

Phenyl acetate: Positive

Strain KB2 (Deposit date: November 15, 1994, Accession No. FERM BP-5354)

A. Gram staining and morphology: Gram-negative rod

B. Growth on various media

Standard agar: Good growth

MacConkey agar: Good growth

C. Optimal temperature for growth: 25°C > 35°C

Growth at 42°C: Good

D. Physiological characteristics

Aerobic/anaerobic: Aerobic

TSI (slant/butt): Alkaline/alkaline, H_2S (-)

Catalase: Positive

Oxidation/fermentation test: -/-

Potassium nitrate reduction: Positive

Indole production from L-tryptophan: Negative

Glucose acidification: Negative

Arginine dehydrase: Negative

Urease: Negative

Esculin hydrolysis (β-glucosidase): Negative

Gelatin hydrolysis (protease): Negative

β-Galactosidase: Negative

Cytochrome oxidase: Positive

E. Assimilation of sugars, organic acids, etc.

Glucose: Negative

L-Arabinose: Negative

D-Mannose: Negative

D-Mannitol: Negative

N-Acetyl-D-glucosamine: Negative

Maltose: Negative

Potassium gluconate: Positive

n-Capric acid: Negative

Adipic acid: Positive

dl-Malic acid: Positive

Sodium citrate: Negative

Phenyl acetate: Negative

Further, in order to exploit the microbial degrading ability more effectively, it is preferable to select the host microorganism for recombinants from the

microorganisms isolated to the environment to be treated, more preferably a dominant microorganism in the environment, considering environmental adaptation of the recombinant. Generally, in the natural world, microorganisms that have existed in an environment will adapt to the environment most probably, and the probability of the survival of foreign microorganisms introduced into the environment is not high. On the other hand, when a very strong microorganism is introduced from outside, it may disturb the existing ecosystem. Thus, the use of the indigenous microorganisms as a host is a superior method in environmental adaptability, survival, and safety.

A transformant to which a recombinant plasmid has been introduced may be cultured in the conditions suitable for the growth of the host. For example, a carbon and nitrogen source such as yeast extract, trypton, and peptone, and a inorganic salt such as sodium chloride and potassium chloride can be used. An M9 medium (containing 6.2 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, and 1.0 g of NH_4Cl in 1 litter) supplemented with various minerals and suitable carbon sources such as sodium malate, sodium succinate, sodium lactate, sodium pyruvate, sodium glutamate, sodium citrate, etc. can also be employed. Further, yeast extract, trypton, peptone, etc. can be used in combination. The pH of the growth medium and culture

temperature can be adjusted to those suitable for the host microorganism, although pH of about 5-9 and culture temperature of 15-37°C are generally preferable.

A transformant containing a recombinant DNA carrying a toluene monooxygenase gene can be suitably employed for the treatment to degrade chlorinated aliphatic hydrocarbon compounds and aromatic compounds (hereinafter referred to as "pollution compounds") contained in a medium. In other words, the degradation treatment for the pollution compounds according to the present invention can be carried out by bringing the transformant into contact with the pollution compounds in an aqueous medium, soil, or a gas phase. Any method can be used to contact the degrading microorganisms with the pollution compounds so long as the microorganisms can express the degrading activity. Various methods such as a batch method, semi-continuous method, and continuous method can be employed. Microorganisms semi-immobilized or immobilized on an appropriate carrier can be also used. The subject such as polluted water, drainage, waste water, soil, and gas phase can be treated by various methods, as required. These treatment methods are described below.

The degradation treatment of the pollution compounds in an aqueous medium according to the present invention can be carried out by contacting the

degrading microorganism with the pollution compounds in the aqueous medium. The representative treating methods are described below. However, the method according to the present invention is not limited thereto, but applicable for any clean-up of the pollution compounds in an aqueous medium.

The simplest method is, for example, to introduce the degrading microorganism directly into an aqueous medium contaminated with the pollution compounds. In this case, it is preferable to optimize the pH, salt concentrations, temperature, and pollutant concentrations of the aqueous medium according to the degrading microorganism.

As another application mode, the degrading microorganism is grown in a culture vessel, and an aqueous medium containing the pollution compounds is introduced into the vessel at a predetermined flow rate to degrade these compounds. The aqueous medium can be introduced and discharged continuously, intermittently or batch-wise according to the treatment capacity. It is preferable to optimize the system by a system control in accordance to the concentrations of the pollution compounds.

Alternatively, the degrading microorganism may be first attached to a carrier such as soil particles and the filled in a reactor vessel, to which an aqueous medium containing the pollution compounds is introduced

for degradation treatment. In this case, any carrier can be employed not restricted to soil particles, but carriers having a high capacity to retain microorganisms and not preventing aeration are preferable. To provide the microorganism with habitats, it can be used various bioreactor carriers, for example, those conventionally employed in the pharmaceutical industry, food industry, and wastewater treatment systems. More specifically, there can be used inorganic particulate carries such as porous glass, ceramics, metal oxides, activated carbon, kaolinite, bentonite, zeolite, silica gel, alumina, and anthracite; gel carries such as starch, agar, chitin, chitosan, polyvinyl alcohol, alginic acid, polyacrylamide, carrageenan, and agarose; ion-exchange cellulose, ion-exchange resins, cellulose derivatives, glutaraldehyde, polyacrylic acid, polyurethane, polyester, or the like. As natural materials, cellulose materials such as cotton, hemp, and papers, and lignin materials such as saw dust and barks can be employed.

The degradation treatment of the pollution compounds in soil according to the present invention can be carried out by bringing the degrading microorganism in contact with the pollution compounds in the soil. The representative treating methods are described below. However, the method according to the

present invention is not limited thereto but applicable to any clean-up of the pollution compounds in soil.

The simplest method is, for example, to introducing degrading microorganisms directly into the soil polluted with the pollution compounds. Introduction of the microorganism may be carried out by spraying it on the surface of the soil and, when the treatment extends to deep underground, by introducing it through the well arranged in the underground, wherein the application of pressure of air, water, etc. allows the microorganism to spread over the wide area of the soil and makes the process more effective. In this case, it is necessary to adjust various conditions of the soil so that they are suitable for the microorganism used for the process.

Another use is such that first the microorganism is attached to a carrier, next the carriers are charged into the reaction vessel, and then the reaction vessel is introduced into, primarily, the aquifer of the contaminated soil, to undergo degradation treatment.

The form of the reaction vessel is desirably like a fence or a film which can cover the wide area of the soil. Any carrier can be used, but it is preferable to use those having an excellent retention of microorganisms and not inhibiting aeration. As a material of the carrier, which can provide suitable habitats for microorganisms, for example, it can be

used various bioreactor carriers, for example, those conventionally employed in the pharmaceutical industry, food industry, and wastewater treatment systems.

According to the present invention, the degradation treatment of the pollution compounds in gas phase can be achieved by contacting the microorganism with the contaminants in the gas phase. The representative modes are shown below, but are not intended to limit the present invention. The present invention is applicable to purification treatment of any gas phase contaminated with the pollution compounds.

One mode is, for example, such that the degradation microorganism is cultured in a culture vessel, and then the gas containing the pollution compounds is introduced into the vessel at a given flow rate to undergo degradation treatment. The method of introducing the gas is not limited specifically, but it is desirably such that introduction of the gas causes agitation of the culture medium and promote its aeration. Introduction and discharge of the gas may be carried out continuously, or it may be carried out intermittently according to the degradation capacity. A batch method is also applicable. Preferably such control is systematized in accordance with the concentrations of the pollution compounds to give optimum results.

Another mode is such that the microorganism is attached to a carrier like soil particles, next the carriers are put into a reaction vessel, and then the gas containing the pollution compounds is introduced into the vessel to undergo degradation treatment. Besides particles of soil, any carrier can be used, however, it is desirable to use those having an excellent retention of microorganisms and not inhibiting aeration. As a material of the carrier, which can provide suitable habitats for microorganisms, for example, it can be used various bioreactor carriers, for example, those conventionally employed in the pharmaceutical industry, food industry, and wastewater treatment systems.

As materials which can retain the degrading microorganism and supply it with nutrient, many examples can be found in the compost used in the agriculture, forestry and fisheries. Specifically, dry materials from plants, such as straw of grains, sawdust, rice bran, bean curd lees, bagasse and so on, and seafood wastes, such as shells of crabs and lobster and so on are applicable.

In purification of contaminated gas, the degrading microorganism may be introduced after the carrier material is packed. To make the degradation reaction efficient, it is preferable that the above-mentioned nutrient, water content, oxygen concentration, etc. are

kept in desirable conditions. The ratio of the carrier to water in a reaction vessel may be determined considering the growth of the microorganism and aeration. The shape of the vessel may be selected considering the amount and concentration of the gas undergoing treatment, but preferably it is designed to enhance the contact of the gas with the microorganism held on the carrier. For example, column, tube, tank and box type are applicable. The vessel of these forms may be joined together with an exhaust duct and a filter to form one unit, or plural vessels may be connected according to the capacity.

Contaminated gas is sometimes adsorbed by the carrier material in the beginning of the reaction and there is very few case where the effect of utilizing microorganism may not be exhibit. After a certain period of time, however, it is thought that the contaminants adhered to the carrier material is degraded, and further contaminants can be adsorbed by the surface of the material to restore adsorption of the material. Thus, a constant decomposition rate is expected without saturation of the pollutant-eliminating ability.

The method according to the present invention is applicable for the treatment of waste liquid, soil and air in a closed system or open system. Moreover, microorganisms may be immobilized on a carrier, or

various methods promoting their proliferation may be employed in combination.

The present invention is explained more specifically by means of the following examples.

<Example 1>

-Cloning of toluene monooxygenase gene of strain KK01-

Cells of strain KK01 (FERM BP-4235) which can assimilate toluene were cultured in 100 ml of LB medium (containing 10 g of trypton, 5 g of yeast extract, and 5 g of sodium chloride in 1 liter) overnight, harvested and washed with 100 mM phosphate buffer (pH 8.0). To the cells thus obtained, 10 ml of STE (10 mM tris (pH 8.0)/1 mM EDTA/100 mM sodium chloride) and 1 ml of 10% sodium dodecyl sulfate (final concentration of about 1%) were added. After the cells were incubated at 70°C for 30 minutes for lysis, phenol treatment and ethanol sedimentation were carried out. DNA thus obtained was dissolved in a 10 mM tris (pH 8.0)/1 mM EDTA buffer (TE).

The DNA thus obtained was dissolved at various concentrations and treated with a restriction enzyme Sau3AI (Takara Shuzo Co., Ltd.) at 37°C for 15 minutes for partial digestion. Aliquots of the partial digestion products were applied to gel electrophoresis on 0.8% agarose gel to identify the samples almost digested to about 5-10 kb. These samples were applied to spin column HR-400 (Amarsham-Pharmacia) to purify

DNA fragments.

The DNA fragments were ligated to plasmid pUC18 (Takara Shuzo Co., Ltd.) completely digested with a restriction enzyme BamHI (Takara Shuzo Co., Ltd.) and dephosphorylated with BAP (Takara Shuzo Co., Ltd.), using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.). Recombinant plasmids thus prepared were then introduced into the host E. coli HB101 (Takara Shuzo Co., Ltd.), and the cells were cultured on LB agar plates containing 100 μg/ml of ampicillin as a selection agent and 200 ppm phenol as an indicator for toluene monooxygenase activity. About 15,000 colonies of transformants grew on the plates.

Eight brown colonies were found in these colonies and picked up. Recombinant plasmid DNA carrying toluene monooxygenase gene was extracted from the cells of each brown colony and the restriction map thereof was determined. It was found that all recombinant plasmids derived from the 8 colonies had a common insertion fragment of 5.8 kb. A plasmid containing only the common fragment of 5.8 kb was designated as pKK01 and a restriction map of the inserted DNA fragment was made (See Fig. 1). A recombinant E.coli HB101 carrying a plasmid containing a 8.5 kb insertion fragment containing this common 5.8 kb fragment was deposited in the National Institute of Bioscience and Human Technology, Agency of Industrial Science and

Technology in accordance with the Budapest Treaty under the accession No. FERM BP-6916. Its microbiological characteristics were identical to those of E. coli HB101 except that it can degrade aromatic compounds and chlorinated aliphatic hydrocarbon compounds.

In order to confirm that the inserted DNA fragment of pKK01 was derived from strain KK01, southern hybridization was performed. DNA was extracted from strain KK01 and completely digested with EcoRI (Takara Shuzo Co., Ltd.) or XhoI (Takara Shuzo Co., Ltd.), and then subjected to southern hybridization. The inserted DNA fragment of pKK01 was digested with BamHI-KpnI (Takara Shuzo Co., Ltd.) to obtain a DNA fragment of about 1.6 kb, and this was used as a probe. As a result, a strong signal was observed around 4.3 kb with the EcoRI-digested DNA, and around 4.2 kb with the XhoI digested DNA, in a good agreement with the lengths of the fragments predicted from the restriction map. Consequently, it was confirmed that the toluene monooxygenase gene contained in pKK01 was derived from the strain KK01.

<Example 2>

-Monooxygenation by E. coli HB101(pKK01)-

The cells of E.coli HB101(pKK01) were inoculated in 100 ml of LB medium, cultured at 37°C overnight, harvested, washed, and then resuspended in 100 ml of M9

medium (6.2 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, and 1.0 g of NH_4Cl per liter) supplemented with a mineral stock solution of the following composition (3 ml/liter of M9 medium)(referred to as M9 + mineral solution).

Composition of mineral stock solution

Nitrilotriacetic acid	:	1.5 g
$MgSO_4$	:	3.0 g
$CaCl_2$	:	0.1 g
Na_2MoO_4	:	0.1 g
$FeSO_4$	:	0.1 g
$MnSO_4$	:	0.5 g
NaCl	:	1.0 g
$ZnSO_4$	:	0.1 g
$CuSO_4$	:	0.1 g
$AlK(SO_4)_2$	:	0.1 g
H_3BO_3	:	0.1 g
$NiCl_2$	:	0.1 g

Distilled water (to 1,000 ml)

Then, 27.5 ml vials were prepared, and 10 ml aliquot of the above suspension was placed in each vial, which was then tightly sealed with a teflon-coated butyl rubber stopper and aluminum seal. Gaseous toluene or benzene was introduced into each vial with a syringe to a concentration of 100 ppm (a concentration supposing all toluene or benzene completely dissolved

in the aqueous phase in the vial). After incubation at 30°C for 3 hours, 1 ml aliquot was taken from each vial, and cells were removed by centrifugation and substances of 10,000 or higher in molecular weight were removed by ultrafiltration. Production of ortho-cresol and 3-methylcatechol from toluene and phenol and catechol from benzene was confirmed by HPLC, to show that toluene and benzene are monooxygenated by toluene monooxygenase encoded by the cloned DNA fragment.

<Example 3>

-Degradation of aromatic compounds and chlorinated aliphatic hydrocarbon compounds by E.coli HB101(pKK01)-

The cells of E.coli HB101(pKK01) cultured as described in Example 2 were suspended in M9 + mineral solution. Ten ml aliquots of the suspension were placed in 27.5 ml vials. Each vial was tightly sealed with a teflon-lined butyl rubber stopper and an aluminum seal. Gaseous trichloroethylene (TCE), cis-1,2-dichloroethylene (cis-1,2-DCE), trans-1,2-dichloroethylene (trans-1,2-DCE), 1,1-dichloroethylene (1,1-DCE), toluene, and benzene were injected into respective vials to a concentration of 5 ppm (a concentration supposing the introduced substance completely dissolved in the aqueous phase in the vial). The vials were shaken and incubated at 30°C. The concentrations of the respective compounds in the gas

phase were measured by gas chromatography after 6 hours. The results are shown in Table 1. E.coli HB101 harboring pUC18 (E.coli HB101(pUC18)) was employed as a control and degradation was evaluated in the same manner.

Another experiment was carried out on TCE degradation in the same manner except that the initial TCE concentration was 10 ppm and when the TCE concentration in the gas phase reached about 0, the process was repeated for total three times. The results are shown in Table 2.

Similarly, phenol, ortho-cresol, meta-cresol and para-cresol were introduced into respective 27.5 ml vials each containing 10 ml of the cell suspension at a concentration of 50 ppm. Each vial was tightly sealed with a butyl rubber stopper and aluminum seal. The vials were shaken and incubated at 30°C. The quantities of the respective compounds in the liquid phase were determined by the amino antipyrine method with a spectrophotometer to obtain their concentrations after 6 hours. The results are shown in Table 2. E.coli HB101(pUC18) was employed as a control and degradation was evaluated in the same manner.

[Table 1]

	E.coliHB101(pKK01)	HB101(pUC18)
TCE	0	5.2
cis-1,2-DCE	0	4.9
trans-1,2-DCE	0	5.1
1,1-DCE	0	5.3
Toluene	0	5.5
Benzene	0	4.9

(Unit: ppm)

[Table 2]

	E.coli HB101(pKK01)	E.coli HB101(pUC18)
Phenol	0	55
Ortho-cresol	0	49
Meta-cresol	0	47
Para-cresol	0	52

(Unit: ppm)

The above results show that E.coli HB101(pKK01) had an excellent ability to degrade aromatic compounds and chlorinated aliphatic hydrocarbon compounds.

<Example 4>

-Definition of toluene monooxygenase region-

The toluene monooxygenase region was defined further by subcloning or stepwise deletion of plasmid pKK01 obtained in Example 1, using restriction sites

thereof. Toluene monooxygenase activity was evaluated by the method in Example 3, and 5 ppm toluene was employed as a substrate.

First, a subclone pKK01 ΔBamHI in which a 0.7-kb fragment was deleted was prepared from pKK01 using the unique BamHI site at 0.7 kb. More specifically, pKK01 was completely digested by restriction enzymes BamHI and HindIII (Takara Shuzo Co., Ltd.) to obtain 2 fragments of 3.4 kb and 5.1 kb. The fragments were separated by agarose gel electrophoresis, and the 5.1 kb fragment was cut out and recovered from the gel and purified with a spin column HR-400 (Amarsham-Pharmacia). The fragment was ligated to pUC18 previously completely digested by BamHI and HindIII enzymes, and E.coli HB101 was transformed with the recombinant plasmids according to the conventional method. E.coli HB101 cells were then applied on an LB plate containing 100 μg/ml of ampicillin to select transformants. From the cells grown overnight in LB medium, plasmid DNA was extracted by an alkaline method to confirm the presence of pKK01ΔBamHI, and a transformant carrying pKK01 ΔBamHI was isolated. E.coli HB101 (pKK01ΔBamHI) cells were evaluated for toluene monooxygenase activity. No degradation of toluene was observed, indicating that the 0.7-kb fragment is essential for toluene monooxygenase activity.

Then, a subclone pKK01ΔEcoRI was prepared by

deleting a 0.3 kb fragment from pKK01 using the 0.3 kb EcoRI restriction site of pKK01. More specifically, pKK01 was partially digested by restriction enzyme EcoRI, and then self-ligated to transform E.coli HB101. The E.coli HB101 transformants were then selected on an LB plate containing 100 μg/ml of ampicillin. After the transformants were cultured in LB medium overnight, the plasmid DNA was extracted from the cells by the alkaline method to confirm the presence of pKK01ΔEcoRI and a transformant carrying pKK01ΔEcoRI was isolated. E, coli HB101(pKK01ΔEcoRI) was evaluated for toluene monooxygenase activity. Degradation of toluene was observed, but the activity was lower than that of E.coli HB101(pKK01), indicating that the 0.3 kb fragment was not essential for toluene monooxygenase activity but necessary for full expression of the activity.

Further, the stepwise deletion method was employed to restrict the toluene monooxygenase region from the opposite direction. More specifically, stepwise deletion was introduced from the XbaI restriction site using XbaI (Takara Shuzo Co., Ltd.) restriction site and Sse8387I (Takara Shuzo Co., Ltd.) restriction site of pUC18. The step-wise deletion was carried out using Deletion Kit for Kilo-Sequence (Takara Shuzo Co., Ltd.) according to the experimental method described in the attached protocol. The results of the activity

evaluation of various deletion clones thus obtained show that the region up to 4.9 kb is essential for expression of the activity and a region from 4.9 kb to 5.8 kb is not especially required for degradation activity.

<Example 5>

-Sequencing of Toluene Monooxygenase Gene-

The nucleotide sequence of pKK01 was determined as follows. pKK01 was digested by various restriction enzymes and subcloned into pUC18 plasmid. Deletion clones were prepared from pKK01 or subclones of partial pKK01 using Deletion Kit for Kilo-Sequence (Takara Shuzo Co., Ltd.) to determine the nucleotide sequence of the 5.8-kb fragment encoding toluene monooxygenase by the dideoxy method. The dideoxy method was carried out using ABI PRISM Cycle Sequencing Kit (Perkin Elmer Corporation) according to the attached protocol for reaction conditions, etc. DNA recombination and Kilo-Sequence method were also performed according to the conventional methods or the manufacturer's protocols attached. The results of sequencing show that the DNA encoding toluene monooxygenase is contained in 5,828 bases comprised of 7 coding regions as shown by SEQ ID NO: 1; a region tomK encoding the amino acid sequence TomK of SEQ ID NO: 2 ; a region TomL encoding the amino acid sequence tomL of SEQ ID NO: 3; a region tomM

encoding an amino acid sequence TomM of SEQ ID NO: 4; a region tomN encoding an amino acid sequence TomN of SEQ ID NO: 5; a region tomO encoding an amino acid sequence (TomO) of SEQ ID NO: 6; a region tomP encoding an amino acid sequence TomP of SEQ ID NO: 7; and a region tomQ encoding an amino acid sequence TomQ of SEQ ID NO: 8.

Here, considering the results of Example 4 together, the polypeptide (TomK)(SEQ ID No: 2) encoded by tomK is not essential for expression of the activity but the presence of TomK clearly enhances the toluene monooxygenase activity. It is therefore desirable for sufficient expression of the activity that TomK is present as a component of toluene monooxygenase. The polypeptide (TomQ)(SEQ ID NO: 8) encoded by tomQ is not essential for expression of the activity. In addition, the toluene monooxygenase activity is not affected by the presence of TomQ. Thus, it is not essential to contain TomQ as a component of toluene monooxygenase.

In other words, any DNA fragment containing segments encoding the amino acid sequences of SEQ ID NOs: 3-7 as the components of toluene monooxygenase where these segments are aligned so that expressed TomL to TomP having the amino acid sequences of SEQ ID NOs: 3-7 can form a protein with a toluene monooxygenase activity is included in the preferred DNA fragment of the present invention. DNA fragments with variation in

at least one segment of the DNA fragment with the proviso that the activity of toluene monooxygenase is not impaired are included in the preferred DNA fragments of the present invention.

DNA fragments further containing a region encoding the amino acid sequence TomK of SEQ ID NO: 2 or a variant in which the amino acid sequence of SEQ ID NO: 2 is changed with the proviso that it does not impair the property to enhance a toluene monooxygenase activity are also included in the preferred embodiment of the present invention.

It should be noted that, in tomK, a sequence corresponding to SD sequence is not found before the 1st ATG (216-218) but present before the 2nd ATG (234-236). Thus, in the following Sequence Listing, the polypeptide encoded by the nucleotide sequence beginning the base number 234 is designated as TomK.

In addition, in tomL, a sequence corresponding to SD sequence is not found before the 1st ATG (bases number 391-393) but present before GTG(463-465). Thus, in the following Sequence Listing, the polypeptide encoded by the nucleotide sequence beginning the base of number 463 is designated as SEQ ID: NO.3 (TomL).

<Example 6>

-Recombination of Toluene Monooxygenase Gene into Expression Vectors-

As expression vectors, pTrc99A (Amarsham-Pharmacia), pSE280 (Invitrogen), and pSE380 (Invitrogen) were employed. They contain an ampicillin-resistant gene as a marker, and pTrc99A has a sequence derived from pBR322, and pSE280 and pSE380 have those derived from ColE1 as ori. All these 3 vectors contain a trc promoter and a rrnB terminator, and a ribosome-binding site is located before the NcoI restriction site. lacIq is contained in pTrc99A and pSE380 but not in pSE280.

To incorporate the toluene monooxygenase gene into these vectors, NcoI restriction sites were introduced in tomK and tomL. The following 5 primers (Takara Shuzo Co., Ltd.) were prepared to introduce the NcoI restriction site by PCR:

SEQ ID NO: 9	tom-K1 5'-AGTCCGCCATGGAGGCGACACCGATCATGAATCAGC-3'	36 mer
SEQ ID NO: 10	tom-K2 5-CACCGACCATGGATCAGCACCCCACCGATCTTTC-3'	34 mer
SEQ ID NO: 11	tom-L1 5'-TGCCGCCTTCCATGGGTTCTGCCGCGAACAGCAG-3'	34 mer
SEQ ID NO: 12	tom-L2 5'-AGCAAGCCATGGCCATCGAGCTGAAGACAGTCGACATCA-3'	39 mer
SEQ ID NO: 13	tail 5'-CCGACCATCACCTGCTCGGCCAGATGGAAGTCGAG-3'	35 mer

The tom-K1 was designed to introduce the NcoI restriction site at the 1st ATG region (bases 216-218 in the Sequence Listing) of tomK. Similarly, tom-K2 was designed to introduce the NcoI site at the 2nd ATG region (bases 234-236 in the SEQ ID NO: 1) of tomK; tomL-1 was designed to introduce the NcoI site at the 1st ATG region (bases 391-393 in SEQ ID NO: 1) of tomL; and tom-L2 was designed to introduce the NcoI site at the 1st GTG region (bases 463-465 in SEQ ID NO: 1) of tomL. Using primer combinations of the primer (5) with the respective primers (1)-(4) and the 8.5 kb fragment-containing plasmid DNA of FERM BP-6916 as the template, PCR was performed. PCR was carried out using Takara LA PCR Kit Ver. 2 (Takara Shuzo Co., Ltd.) with a reaction volume of 50 µl, repeating 30 times a cycle of reaction at 94°C for 1 minute and 98°C for 20 seconds followed by 72°C for 5 minutes (shuttle PCR), then followed by reaction at 72°C for 10 minutes. The reaction conditions were according to the manufacturer's protocol.

As a result, the combinations of the primers (1) and (5), (2) and (5), (3) and (5), and (4) and (5) gave the PCR products of about 5.6 kb, about 5.6 kb, about 5.4 kb, and about 5.4 kb, respectively. The respective DNA fragments were digested with the restriction enzyme NcoI (Takara Shuzo Co., Ltd.) to give the respective fragments of about 5.0 kb, about 5.0 kb, about 4.9 kb,

and about 4.8 kb together with a fragment of about 0.6 kb. It shows that PCR products were completely digested by the restriction enzyme NcoI. These NcoI-digested products were purified using a spin column HR-4000 (Amarsham-Pharmacia) and used for the following ligation reaction.

The above expression vectors were completely digested with the restriction enzyme NcoI, dephosphorylated, subjected to phenol treatment, and purified with a spin column HR-400 (Amarsham-Pharmacia). The vectors were then ligated to the NcoI-digested PCR products to transform E.coli HB101 (Takara Shuzo Co., Ltd.) according to the conventional method. The transformed E.coli HB101 cells were then grown on LB plate containing 100 μg/ml of ampicillin for transformant selection. After the transformants were cultured in LB medium at 37°C overnight, plasmid DNA was extracted by the alkaline method to examine the recombinant plasmids. Transformants in which the respective PCR fragments were accurately inserted into the NcoI restriction site of the respective expression vectors were obtained.

A list of the obtained recombinant plasmids are shown in Table 3.

[Table 3]

	tom-K1	tom-K2	tom-L1	tom-L2
pTrc99A	pK19	pK29	pL19	pL29
pSE280	pK12	pK22	pL12	pL22
pSE380	pK13	pK23	pL13	pL23

<Example 7>

-Ability of E.coli HB101 Recombinant Strains to Degrade Aromatic Compounds and Chlorinated Aliphatic Hydrocarbon Compounds (without Induction with IPTG)-

The cells of the E.coli strains, each harboring one of the 12 recombinant plasmids obtained as described in Example 6, were inoculated in 100 ml of LB medium, cultured at 37°C overnight, harvested, washed, and suspended in an M9 + mineral solution. Ten ml aliquots of the suspension were placed in 27.5 ml vials, and each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminium seal. Then, gaseous trichloroethylene (TCE), cis-1,2-dichloroethylene (cis-1,2-DCE), trans-1,2-dichloroethylene (trans-1,2-DCE), 1,1-dichloroethylene (1,1-DCE), toluene, and benzene were added to respective vials with a syringe to a concentration of 20 ppm (supposing all of the introduced substance

dissolved in the aqueous phase in the vial). The vials were shaken and incubated at 30°C. The concentrations of the respective compounds in the gas phase after 6 hour incubation were measured by gas chromatography. The results are shown in Table 4. E.coli HB101(pSE280) was employed as a control and degradation was evaluated in the same manner.

[Table 4]

	pK19	pK29	pL19	pL29	pK12	pK22	pL12	pL22
TCE	4.5	5.2	7.8	7.5	0	0	0.4	0.2
cis-1,2-DCE	2.5	2.4	3.8	4.5	0	0	2.1	3.2
trans-1,2-DCE	3.1	4.2	5.2	5.8	0	0	1.5	1.4
1,1-DCE	7.2	6.6	8.9	9.1	0	0	1.2	0.9
Toluene	1.3	1.1	2.5	3.2	0	0	0	0
Benzene	4.8	5.1	7.3	6.8	0	0	0.9	0.5

	pK13	pK23	pL13	pL23	pSE280
TCE	3.8	4.3	5.5	5.3	20.1
cis-1,2-DCE	0.9	0.7	1.5	1.8	18.9
trans-1,2-DCE	1.2	1.1	2.1	2.1	19.8
1,1-DCE	2.5	2.4	5.1	4.9	20.7
Toluene	1.2	0.9	1.8	1.7	21.0
Benzene	3.5	3.3	4.8	4.4	20.2

(Unit: ppm)

Similarly, phenol, ortho-cresol, meta-cresol and para-cresol were introduced into respective 27.5 ml vials each containing 10 ml of the cell suspension at a concentration of 50 ppm. Each vial was tightly sealed with a butyl rubber stopper and aluminum seal. The vials were shaken and incubated at 30°C. The quantities of the respective compounds in the liquid

phase were determined by the amino antipyrine method with a spectrophotometer to determine their concentrations after 6 hours. The results are shown in Table 5. E.coli HB101(pSE280) was employed as a control and degradation was evaluated in the same manner.

[Table 5]

	pK19	pK29	pL19	pL29	pK12	pK22	pL12	pL22
Phenol	0	0	0	0	0	0	0	0
Ortho-cresol	0	0	0	0	0	0	0	0
Meta-cresol	0	0	0	0	0	0	0	0
Para-cresol	0	0	0	0	0	0	0	0

	pK13	pK23	pL13	pL23	pSE280
Phenol	0	0	0	0	50.6
Ortho-cresol	0	0	0	0	52.5
Meta-cresol	0	0	0	0	53.1
Para-cresol	0	0	0	0	50.5

(Unit: ppm)

The above results confirm that E.coli HB101 transformants harboring the expression vectors have an excellent ability to degrade the aromatic compounds and chlorinated aliphatic hydrocarbon compounds. It is shown that transformants harboring pTrc99A or pSE380-derived expression vectors express a lower degrading activity in a system not containing IPTG than those harboring pSE280-derived plasmids, since pSE280 lacks lacIq.

<Example 8>

-Ability of E.coli HB101 Transformants harboring Expression Vectors to Degrade Aromatic Compounds and Chlorinated Aliphatic Hydrocarbon Compounds (with Induction with IPTG)-

Each E.coli HB101 transformant strain harboring one of the 12 recombinant plasmids obtained as described in Example 6, was inoculated in 100 ml of LB medium, cultured at 37°C to reach OD_{600} of about 0.8, and then IPTG was added to 1 mM concentration followed by further incubation at 37°C for 5 hours. Then the cells were harvested, washed and suspended in an M9 + mineral solution. Ten ml aliquots of the suspension were placed in 27.5 ml vials, and each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminium seal. Then, gaseous trichloroethylene (TCE), cis-1,2-dichloroethylene (cis-1,2-DCE), trans-1,2-

dichloroethylene (trans-1,2-DCE), 1,1-dichloroethylene (1,1-DCE), toluene, and benzene were added to respective vials with a syringe to a concentration of 20 ppm (supposing all of the introduced substance dissolved in the aqueous phase in the vial). The vials were shaken and incubated at 30°C. The concentrations of the respective compounds in the gas phase after 6 hour incubation were measured by gas chromatography. The results are shown in Table 6. E.coli HB101(pSE280) was employed as a control and degradation was evaluated in the same manner.

[Table 6]

	pK19	pK29	pL19	pL29	pK12	pK22	pL12	pL22
TCE	0	0	0	0	0	0	0.7	0.5
cis-1,2-DCE	0	0	0	0	0	0	1.9	2.1
trans-1,2-DCE	0	0	0	0	0	0	0.9	1.9
1,1-DCE	0	0	0.7	0.5	0	0	0.8	0.7
Toluene	0	0	0	0	0	0	0	0
Benzene	0	0	1.2	2.1	0	0	1.3	0.9

	pK13	pK23	pL13	pL23	pSE280
TCE	0	0	0	0	21.2
cis-1,2-DCE	0	0	0	0	19.9
trans-1,2-DCE	0	0	0	0	20.7
1,1-DCE	0	0	0	0	19.8
Toluene	0	0	0	0	20.5
Benzene	0	0	0.3	0.1	21.0

(Unit: ppm)

Similarly, phenol, ortho-cresol, meta-cresol and para-cresol were introduced into respective 27.5 ml vials each containing 10 ml of the cell suspension, at a concentration of 50 ppm. Each vial was tightly sealed with a butyl rubber stopper and aluminum seal. The vials were shaken and incubated at 30°C. The quantities of the respective compounds in the liquid phase were determined by the amino antipyrine method with a spectrophotometer to determine their concentrations after 6 hours. The results are shown in Table 7. E.coli HB101(pSE280) was employed as a control and degradation was evaluated in the same manner.

[Table 7]

	pK19	pK29	pL19	pL29	pK12	pK22	pL12	pL22
Phenol	0	0	0	0	0	0	0	0
Ortho-cresol	0	0	0	0	0	0	0	0
Meta-cresol	0	0	0	0	0	0	0	0
Para-cresol	0	0	0	0	0	0	0	0

	pK13	pK23	pL13	pL23	pSE280
Phenol	0	0	0	0	50.0
Ortho-cresol	0	0	0	0	51.1
Meta-cresol	0	0	0	0	52.3
Para-cresol	0	0	0	0	47.9

(Unit: ppm)

The above results confirm that E.coli HB101 transformants harboring toluene monooxygenase-expression vectors has an excellent ability to degrade aromatic compounds and chlorinated aliphatic hydrocarbon compounds. It is shown that transformants harboring pTrc99A- or pSE380-based expression vectors show more excellent degrading activity by IPTG

induction.

<Example 9>

-TCE Degradation by E.coli HB101(pK22) and HB101(pK23) recombinant Strains in Soil (Without IPTG Induction)-

E.coli HB101(pK22) and HB101(pK23) recombinant strains as described in Example 6 were respectively inoculated in 10 ml of LB medium and cultured at 37°C overnight. Fifty grams of Sawara sieved sand (unsterilized) was placed in 68 ml vials each. Five ml of LB medium inoculated with the above seed culture to 100:1, was then added to the sand in each vial. Each vial was cotton-plugged, and incubated at 37°C for 8 hours without shaking. After that, each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal. Gaseous TCE was introduced into the vials with a syringe to 20 ppm (supposing all TCE dissolved into the aqueous phase in the vial). The vials were incubated at 30°C. Quantitative analysis of TCE in the gas phase were carried out by gas chromatography after 6 hours to determine TCE concentrations. The results are shown in Table 8. E.coli HB101(pSE280) was employed as a control and evaluated in the same manner.

[Table 8]

	pK22	pK23	pSE280
TCE	0	2.4	20.8

(Unit: ppm)

The above results confirm that E.coli HB101 transformants harboring pK22 and pK23 also show an excellent TCE-degrading ability in soil. It is shown that transformant harboring pK23 (pSE380-based) expresses a lower degrading activity in a system not containing IPTG than that harboring pSE280-derived plasmid pK23, since the former contains lacIq.

<Example 10>

-TCE Degradation by E.coli HB101(pK22) or HB101(pK23) in Soil (With IPTG Induction)-

The cells of E.coli HB101(pK22) and HB101(pK23) recombinant strains as described in Example 6 were respectively inoculated in 10 ml of LB medium and cultured at 37°C overnight. Fifty grams of Sawara sieved sand (unsterilized) were placed in 68 ml vials each. Five ml of LB medium inoculated with the above seed culture to 100:1, was then added to the sand. Each vial was cotton-plugged, and incubated at 37°C for

4 hours without shaking. Then 1 ml of a 10 mM IPTG solution was added to each vial. After that, each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal. Gaseous TCE was introduced into the vials with a syringe to 20 ppm (supposing all TCE dissolved into the aqueous phase in the vial). The vials were incubated at 30°C. Quantitative analysis of TCE in the gas phase were carried out by gas chromatography after 6 hours to determine TCE concentrations. The results are shown in Table 9. E.coli HB101(pSE280) was employed as a control and evaluated in the same manner.

[Table 9]

	pK22	pK23	pSE280
TCE	0	0	20.3

(Unit: ppm)

The above results confirm that E.coli HB101 transformants harboring pK22 and pK23 also show an excellent TCE-degrading ability in soil. It is shown that transformant harboring pK23 (pSE380-based) expresses higher degrading activity with IPTG induction.

<Example 11>

-TCE Degradation by E.coli HB101(pK22) or HB101(pK23) in Gas Phase (Without IPTG Induction)-

The cells of respective recombinant strains, E.coli HB101(pK22) and HB101(pK23) as described in Example 6, were inoculated in 100 ml of LB medium and cultured at 37°C overnight. Aliquots (30 ml) of each seed culture were transferred into 68 ml vials, into which air which had passed through a saturation TCE solution was introduced at a flow rate of 20 ml/min for 10 minutes. Each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal, and shaking culture was conducted at 30°C. Quantitative analysis of TCE in the gas phase were carried out by gas chromatography to determine its concentration after 6 hours. The results are shown in Table 10. E.coli HB101(pSE280) was employed as a control and degradation was evaluated in the same manner.

[Table 10]

	pK22	pK23	pSE280
TCE	0	12.1	47.9

(Unit: ppm)

The above results confirm that recombinant E.coli HB101(pK22) or HB101(pK23) shows an excellent TCE-degrading ability also in the gas phase. It is shown that transformant harboring pK23 (pSE380-based) expresses a lower degrading activity in a system not containing IPTG than that harboring pSE280-derived plasmid pK23, since the former contains lacIq.

<Example 12>

-TCE Degradation by recombinant E.coli HB101(pK22) and HB101(pK23) in Gas Phase (With IPTG Induction)-

E.coli (HB101) recombinant strains each harboring pK22 or pK23 as described in Example 6 were respectively inoculated into 100 ml of LB medium and cultured at 37°C to reach OD_{600} of about 0.8, and then IPTG was added to 1 mM concentration followed by further incubation at 37°C for 5 hours. Aliquots (30 ml) of the cell suspension were transferred into 68 ml vials, into which air which had passed through in a saturated TCE solution was introduced at a flow rate of 20 ml/min for 10 minutes. Each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal, and shaking culture was conducted at 30°C. Quantitative analysis of TCE in the gas phase were carried out by gas chromatography to determine its concentration after 6 hours. The results are shown in Table 11. E.coli HB101(pSE280) was employed as a

control and degradation was evaluated in the same manner.

[Table 11]

	pK22	pK23	pSE280
TCE	0	0	54.2

(Unit: ppm)

The above results confirm that recombinant E.coli HB101(pK22) or HB101(pK23) shows an excellent TCE-degrading ability also in the gas phase, and show that transformant harboring pK23 (pSE380-based) expresses higher degrading activity with IPTG induction.

<Example 13>

-Introduction of Recombinant Plasmid containing Toluene Monooxygenase Gene into Vibrio sp. strain KB1-

The toluene monooxygenase gene beginning from the second ATG of tomK (base number 234-236) was transferred from the recombinant plasmid pK29 of Example 6 (recombinant pTrc99A containing the gene) into a vector pBBR122 (Mo Bi Tec) having a wide host range replication region not belonging to an incompatible group of IncP, IncQ, and IncW. This

recombinant plasmid was introduced in Vibrio sp. strain KB1, and its ability to degrade aromatic compounds and chlorinated aliphatic hydrocarbon compounds was evaluated.

First, a wide host range recombinant plasmid was constructed. An about 7.0-kb fragment containing the toluene monooxygenase gene, a trc promoter, and a rrnB terminator was cut out from pK29 using the restriction enzymes HpaI (Takara Shuzo Co., Ltd.) and SmaI (Takara Shuzo Co., Ltd.). This fragment of about 7.0 kb does not contain the lacIq sequence. As a vector of a wide host range, pBBR122 was employed. pBBR122 was completely digested with the restriction enzyme SmaI (Takara Shuzo Co., Ltd.). The 7.0 kb fragment containing the toluene monooxygenase gene, a trc promoter, and an rrnB terminator prepared as described above was ligated to the SmaI restriction site of the pBBR122 using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.) and the recombinant plasmid thus constructed was introduced into E.coli HB101 (Takara Shuzo Co., Ltd.). The cells of the E.coli thus treated were applied on LB plate containing 50 µg/ml of chloramphenicol as a selection agent. When the colonies on the plate grew to an appropriate size, the colonies were transferred by replica printing onto an LB plate containing 50 µg/ml of kanamycin as a selection agent. Transformants that could proliferate

on the plate with chloramphenicol but not on the plate with kanamycin were selected, and cultured in LB medium at 37°C overnight, to extract plasmid DNA from the cells by the alkaline method. After checking the plasmids, transformants harboring a recombinant plasmid where the 7.0 kb fragment was correctly inserted into the SmaI site of the pBBR122 were obtained. The recombinant plasmid thus obtained was about 12.3 kb in length and designated as pK29bbr.

The SOB medium shown below was employed for liquid culture of Vibrio sp. strain KB1. Chloramphenicol was used at a concentration of 50 μg/ml as a selection agent and the culture temperature was 30°C. The recombinant plasmid pK29 was introduced into Vibrio sp. strain KB1 cells by electroporation using a gene pulsar (Bio-Rad). The recombinant plasmid pK29bbr was stably retained after introduction into Vibrio sp. strain KB1.

SOB medium:

Trypton: 20 g

Yeast extract: 5 g

NaCl: 0.5 g

250 mM KCl: 10 ml

Distilled water (to 990 ml)

pH 7.0

The above solution was sterilized by autoclaving and cooled to room temperature, to which 10 ml of a 2 M Mg solution (1 M $MgSO_{4.7}H_2O$ + 1 M $MgCl_{2.6}H_2O$) separately

sterilized by autoclaving was added.

<Example 14>

-Ability of Vibrio sp. KB1(pK29bbr) to Degrade Aromatic Compounds and Chlorinated Aliphatic Hydrocarbon Compounds-

The cells of Vibrio sp. KB1(pK29bbr) were inoculated in 100 ml of SOB medium, cultured at 30°C overnight, harvested, washed, and then suspended in 100 ml of M9 (containing 6.2 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, and 1.0 g of NH_4Cl per liter) supplemented with a mineral stock solution (3 ml to 1 liter of M9 medium).

Ten ml of the suspension was placed in respective 27.5 ml vials and each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal. Then, gaseous trichloroethylene (TCE), cis-1,2-dichloroethylene (cis-1,2-DCE), trans-1,2-dichloroethylene (trans-1,2-DCE), 1,1-dichloroethylene (1,1-DCE), toluene, and benzene were added to respective vials with a syringe to a concentration of 20 ppm (supposing all of the introduced substance dissolved in the aqueous phase in the vial). The vials were shaken and incubated at 30°C. The concentrations of the respective compounds in the gas phase after 6 hour incubation were measured by gas chromatography. The results are shown in Table 12. Vibrio sp.

KB1(pBBR122) was tested as a control and degradation was evaluated in the same manner.

[Table 12]

	KB1(pK29bbr)	KB1(pBBR122)
TCE	0	19.1
cis-1,2-DCE	0	20.2
trans-1,2-DCE	0	21.3
1-1,DCE	0	19.2
Toluene	0	19.8
Benzene	0	21.0

(Unit: ppm)

Similarly, to 10 ml of the prepared cell suspension in a 27.5-ml vial, phenol, ortho-cresol, meta-cresol, and para-cresol were added to 50 ppm, respectively. The vial was tightly sealed with a butyl rubber stopper and aluminum seal, and then shaken and incubated at 30°C. The quantities of the respective compounds in the liquid phase were measured by the amino antipyrine method with a spectrophotometer to obtain their concentrations after 6 hours. The results are shown in Table 13. Vibrio species strain KB1 containing only pBBR122 was employed as a control and

degradation was evaluated in a similar system.

Similarly, phenol, ortho-cresol, meta-cresol and para-cresol were introduced at a concentration of 50 ppm into respective 27.5 ml vials each containing 10 ml of the cell suspension. Each vial was tightly sealed with a butyl rubber stopper and aluminum seal, and shaken and incubated at 30°C. The quantities of the respective compounds in the liquid phase were determined by the amino antipyrine method using a spectrophotometer to determine their concentrations after 6 hours. The results are shown in Table 13. Vibrio sp. KB1(pBBR122) was tested as a control and degradation was evaluated in the same manner.

[Table 13]

	KB1(pK29bbr)	KB1(pBBR122)
Phenol	0	51
Ortho-cresol	0	50
Meta-cresol	0	49
Para-cresol	0	50

(Unit: ppm)

The above results show that the recombinant Vibrio

sp. strain KB1 harboring pK29bbr can constitutively express the ability to degrade aromatic compounds and chlorinated aliphatic hydrocarbon compounds.

<Example 15>

-Degradation of TCE by Recombinant Vibrio sp. KB1(pK29bbr) in Soil-

Vibrio sp. KB1(pK29bbr) recombinant strain as described in Example 13 was inoculated in 10 ml of SOB medium and cultured at 30°C overnight. Fifty grams of Sawara sieved sand (unsterilized) was placed in each 68 ml vial. Five ml of SOB medium inoculated with the above seed culture to 100:1 was then added to the sand in each vial. Each vial was cotton-plugged and incubated at 30°C for 12 hours without shaking. After that, each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal. Gaseous TCE was introduced into the vials with a syringe to 20 ppm (supposing all TCE dissolved into the aqueous phase in the vial). The vials were incubated at 30°C. Quantitative analysis of TCE in the gas phase were carried out by gas chromatography after 6 hours to determine TCE concentrations. The results are shown in Table 14. Vibrio sp. KB1(pBBR122) was tested as a control and degradation was evaluated in the same manner.

[Table 14]

	KB1(pK29bbr)	KB1(pBBR122)
TCE	0	20.2

(Unit: ppm)

The above results show that the recombinant Vibrio sp. KB1(pK29bbr) can constitutively express the ability to degrade TCE also in soil.

<Example 16>

-Degradation of TCE by Recombinant Vibrio sp. KB1(pK29bbr) in Gas Phase-

The cells of recombinant Vibrio sp. KB1(pK29bbr) as described in Example 13 were inoculated in 100 ml of SOB medium and cultured at 30°C overnight. Aliquots (30 ml) of the seed culture were transferred into 68 ml vials, into which air which had passed through a saturation TCE solution was introduced at a flow rate of 20 ml/min for 10 minutes. Each vial was tightly sealed with a Teflon-coated butyl rubber stopper and aluminum seal, and shaking culture was conducted at 30°C. Quantitative analysis of TCE in the gas phase were carried out by gas chromatography to determine its concentration after 6 hours. The results are shown in Table 15. Vibrio sp. KB1(pBBR122) was employed as a

control and degradation was evaluated in the same manner.

[Table 15]

	KB1(pK29bbr)	KB1(pBBR122)
TCE	0	52.1

(Unit: ppm)

The above results show that the recombinant Vibrio sp. KB1(pK29bbr) can constitutively express the ability to degrade TCE also in the gas phase.

According to the present invention, a DNA fragment carrying a toluene monooxygenase gene with an excellent ability to degrade aromatic compounds and chlorinated aliphatic hydrocarbon compounds can be obtained. In addition, a novel recombinant plasmid containing the DNA fragment as a whole or a part thereof that can be utilized to obtain a transformant capable of degrading aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds can be obtained. Further, a transformant harboring the plasmid and can be utilized to degrade aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds can be obtained. Furthermore, a practical method for environmental

remediation that can efficiently degrade either aromatic compounds and/or chlorinated aliphatic hydrocarbon compounds by utilizing the transformant.